a similar fashion using a 5' phosphorylated primer having the sequence

5'-pGGC-GTT-GCG-CCA-*TGC-GCA-TCA-CT-3'.

(The asterisk indicates the position of the mismatch and the underlined sequence shows the position of a new MstI site.) The C24 and C87 mutations were obtained at a frequency of one and two percent, respectively. Mutant sequences were confirmed by dideoxy sequencing in M13.

Mutagenesis of Tyr21/Thr22 to A21/C22 was carried out with a 5' phosphorylated oligonucleotide primer having the sequence

5'-pAC-TCT-CAA-GGC-***GCT-**TGT-GGC-TCA-AAT-GTT-3'.

(The asterisks show mismatches to the wild type sequence and the underlined sequence shows the position of an altered Sau3A site.) Manipulations for heteroduplex synthesis were identical to those described for C24. Because direct cloning of the heteroduplex DNA fragment can yield increased frequencies of mutagenesis, the EcoRI-BamHI subtilisin fragment was purified and ligated into pBS42. E. coli MM 294 cells were transformed with the ligation mixture and plasmid DNA was purified from isolated transformants. Plasmid DNA was screened for the loss of the Sau3A site at codon 23 that was eliminated by the mutagenesis primer. Two out of 16 plasmid preparations had lost the wild type Sau3A site. The mutant sequence was confirmed by dideoxy sequencing in M13.

Double mutants, C22/C87 and C24/C87, were constructed by ligating fragments sharing a common ClaI site that separated the single parent cystine codons. Specifically, the 500 bp EcoRI-ClaI fragment containing the 5' portion of the subtilisin gene (including codons 22 and 24) was ligated with the 4.7 kb ClaI-EcoRI fragment that contained the 3' portion of the subtilisin gene (including codon 87) plus pBS42 vector sequence. E. coli MM 294 was transformed with ligation mixtures and plasmid DNA was purified from individual transformants. Double-cysteine plasmid constructions were identified by restriction site markers originating from the parent cysteine mutants (i.e., C22 and C24, Sau3A minus; Cys87, MstI plus). Plasmids from E. coli were transformed into B. subtilis BG2036. The thermal stability of these mutants as compared to wild type subtilisin are presented in Figure 30 and Tables XVII and XVIII.

TABLE XVII

Effect of DTT on the Half-Time of Autolytic Inactivation of Wild-Type and Disulfide Mutants of Subtilisin*

Enzyme	t_i -DDT	t_i +DTT	-DTT/+DTT
	min		
Wild-type	95	85	1.1
C22/C87	44	25	1.8
C24/C87	92	62	1.5

(*) Purified enzymes were either treated or not treated with 25mM DTT and dialyzed with or without 10mM DTT in 2mM $CaCl_2$, 50mM Tris (pH 7.5) for 14 hr. at 4°C. Enzyme concentrations were adjusted to 80µl aliquots were quenched on ice and assayed for residual activity. Half-times for autolytic inactivation were determined from semi-log plots of $\log_{10}$ (residual activity) versus time. These plots were linear for over 90% of the inactivation.

TABLE XVIII

Effect of Mutations in Subtilisin on the Half-Time of Autolytic Inactivation at 58°C*

Enzyme	$t_{\frac{1}{2}}$ min
Wild-type	120
C22	22
C24	120
C87	104
C22/C87	43
C24/C87	115

(*) Half-times for autolytic inactivation were determined for wild-type and mutant subtilisins as described in the legend to Table III. Unpurified and non-reduced enzymes were used directly from B. subtilis culture supernatants.

The disulfides introduced into subtilisin did not improve the autolytic stability of the mutant enzymes when compared to the wild-type enzyme. However, the disulfide bonds did provide a margin of autolytic stability when compared to their corresponding reduced double-cysteine enzyme. Inspection of a highly refined x-ray structure of wild-type B. amyloliquefaciens subtilisin reveals a hydrogen bond between Thr22 and Ser87. Because cysteine is a poor hydrogen donor or acceptor (Paul, I.C. (1974) in Chemistry of the -SH Group (Patai, S., ed.) pp. 111-149, Wiley Interscience, New York) weakening of 22/87 hydrogen bond may explain why the C22 and C87 single-cysteine mutant proteins are less autolytically stable than either C24 or wild-type (Table XVIII). The fact that C22 is less autolytically stable than C87 may be the result of the Tyr21A mutation (Table XVIII). Indeed,

construction and analysis of Tyr21/C22 shows the mutant protein has an autolytic stability closer to that of C87. In summary, the C22 and C87 of single-cysteine mutations destabilize the protein toward autolysis, and disulfide bond formation increases the stability to a level less than or equal to that of wild-type enzyme.

EXAMPLE 12

Multiple Mutants Containing Substitutions at Position 222 and Position 166 or 169

Double mutants 166/222 and 169/222 were prepared by ligating together (1) the 2.3kb AcaII fragment from pS4.5 which contains the 5' portion of the subtilisin gene and vector sequences, (2) the 200bp AvaII fragment which contains the relevant 166 or 169 mutations from the respective 166 or 169 plasmids, and (3) the 2.2kb AvaII fragment which contains the relevant 222 mutation 3' and of the subtilisin genes and vector sequence from the respective p222 plasmid.

Although mutations at position 222 improve oxidation stability they also tend to increase the Km. An example is shown in Table XIX. In this case the A222 mutation was combined with the K166 mutation to give an enzyme with kcat and Km intermediate between the two parent enzymes.

TABLE XIX

	Kcat	Km
WT	50	1.4×10^{-4}
A222	42	9.9×10^{-4}
K166	21	3.7×10^{-5}
K166/A222	29	2.0×10^{-4}

substrate sAAPFpNa

EXAMPLE 13

Multiple Mutants Containing Substitutions at Positions 50, 156, 166, 217 and Combinations Thereof

The double mutant S156/A169 was prepared by ligation of two fragments, each containing one of the relevant mutations. The plasmid pS156 was cut with XmaI and treated with S1 nuclease to create a blunt end at codon 167. After removal of the nuclease by phenol/chloroform extraction and ethanol precipitation, the DNA was digested with BamHI and the approximately 4kb fragment containing the vector plus the 5' portion of the subtilisin gene through codon 167 was purified.

The pA169 plasmid was digested with KpnI and treated with DNA polymerase Klenow fragment plus 50 μM dNTPs to create a blunt end codon at codon 168. The Klenow was removed by phenol/chloroform extraction and ethanol precipitation. The DNA was digested with BamHI and the 590bp fragment including codon 168 through the carboxy terminus of the subtilisin gene

was isolated. The two fragments were then ligated to give S156/A169.

Triple and quadruple mutants were prepared by ligating together (1) the 220bp PvuII/HaeII fragment containing the relevant 156, 166 and/or 169 mutations from the respective p156, p166 and/or p169 double of single mutant plasmid, (2) the 550bp HaeII/BamHI fragment containing the relevant 217 mutant from the respective p217 plasmid, and (3) the 3.9kb PvuII/BamHI fragment containing the F50 mutation and vector sequences.

The multiple mutant F50/S156/A169/L217, as well as B. amyloliquefaciens subtilisin, B. lichenformis subtilisin and the single mutant L217 were analyzed with the above synthetic polypeptides where the P-1 amino acid in the substrate was Lys, His, Ala, Gln, Tyr, Phe, Met and Leu. These results are shown in Figures 26 and 27.

These results show that the F50/S156/A169/L217 mutant has substrate specificity similar to that of the B. licheniformis enzyme and differs dramatically from the wild type enzyme. Although only data for the L217 mutant are shown, none of the single mutants (e.g., F50, S156 or A169) showed this effect. Although B. licheniformis differs in 88 residue positions from B. amyloliquefaciens, the combination of only these four mutations accounts for most of the differences in substrate specificity between the two enzymes.

EXAMPLE 14

Subtilisin Mutants Having Altered Alkaline Stability

A random mutagenesis technique was used to generate single and multiple mutations within the B.

amyloliquefaciens subtilisin gene. Such mutants were screened for altered alkaline stability. Clones having increased (positive) alkaline stability and decreased (negative) alkaline stability were isolated and sequenced to identify the mutations within the subtilisin gene. Among the positive clones, the mutants V107 and R213 were identified. These single mutants were subsequently combined to produce the mutant V107/R213.

One of the negative clones (V50) from the random mutagenesis experiments resulted in a marked decrease in alkaline stability. Another mutant (P50) was analyzed for alkaline stability to determine the effect of a different substitution at position 50. The P50 mutant was found to have a greater alkaline stability than wild type subtilisin and when combined with the double mutant V107/R213 resulted in a mutant having an alkaline stability which reflected the aggregate of the alkaline stabilities for each of the individual mutants.

The single mutant R204 and double mutant C204/R213 were identified by alkaline screening after random cassette mutagenesis over the region from position 197 to 228. The C204/R213 mutant was thereafter modified to produce mutants containing the individual mutations C204 and R213 to determine the contribution of each of the individual mutations. Cassette mutagenesis using pooled oligonucleotides to substitute all amino acids at position 204, was utilized to determine which substitution at position 204 would maximize the increase in alkaline stability. The mutation from Lys213 to Arg was maintained constant for each of these substitutions at position 204.

A. Construction of pB0180, an *E. coli-B. subtilis* Shuttle Plasmid

The 2.9 kb EcoRI-BamHI fragment from pBR327 (Covarrubias, L., et al. (1981) Gene 13, 25-35) was ligated to the 3.7kb EcoRI-BamHI fragment of pBD64 (Gryczan, T., et al. (1980) J. Bacteriol., 141, 246-253) to give the recombinant plasmid pB0153. The unique EcoRI recognition sequence in pBD64 was eliminated by digestion with EcoRI followed by treatment with Klenow and deoxynucleotide triphosphates (Maniatis, T., et al. (eds.) (1982) in Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.). Blunt end ligation and transformation yielded pB0154. The unique AvaI recognition sequence in pB0154 was eliminated in a similar manner to yield pB0171. pB0171 was digested with BamHI and PvuII and treated with Klenow and deoxynucleotide triphosphates to create blunt ends. The 6.4 kb fragment was purified, ligated and transformed into LE392 cells (Enquest, L.W., et al. (1977) J. Mol. Biol. 111, 97-120), to yield pB0172 which retains the unique BamHI site. To facilitate subcloning of subtilisin mutants, a unique and silent KpnI site starting at codon 166 was introduced into the subtilisin gene from pS4.5 (Wells, J.A., et al. (1983) Nucleic Acids Res., 11, 7911-7925) by site-directed mutagenesis. The KpnI+ plasmid was digested with EcoRI and treated with Klenow and deoxynucleotide triphosphates to create a blunt end. The Klenow was inactivated by heating for 20 min at 68°C, and the DNA was digested with BamHI. The 1.5 kb blunt EcoRI-BamHI fragment containing the entire subtilisin was ligated with the 5.8 kb NruI-BamHI from pB0172 to yield pB0180. The ligation of the blunt NruI end to the blunt EcoRI end recreated an EcoRI

site. Proceeding clockwise around pB0180 from the EcoRI site at the 5' end of the subtilisin gene is the unique BamHI site at the 3' end of the subtilisin gene, the chloramphenicol and neomycin resistance genes and UB110 gram positive replication origin derived from pBD64, the ampicillin resistance gene and gram negative replication origin derived from pBR327.

B. Construction of Random Mutagenesis Library

The 1.5 kb EcoRI-BamHI fragment containing the B. amyloliquefaciens subtilisin gene (Wells et al., 1983) from pB0180 was cloned into M13mp11 to give M13mp11 SUBT essentially as previously described (Wells, J.A., et al. (1986) J. Biol. Chem., 261,6564-6570). Deoxyuridine containing template DNA was prepared according to Kunkel (Kunkel, T.A. (1985) Proc. Natl. Acad. Sci. USA, 82 488-492). Uridine containing template DNA (Kunkel, 1985) was purified by CsCl density gradients (Maniatis, T. et al. (eds.) (1982) in Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.). A primer (AvaI$^-$) having the sequence

5'GAAAAAAGACCC$\overset{*}{T}$AGCGTCGCTTA

ending at codon -11, was used to alter the unique AvaI recognition sequence within the subtilisin gene. (The asterisk denotes the mismatches from the wild-type sequence and underlined is the altered AvaI site.)

The 5' phosphorylated AvaI primer (~320 pmol) and ~40 pmol (~120µg) of uridine containing M13mp11 SUBT template in 1.88 ml of 53 mM NaCl, 7.4 mM MgCl2 and 7.4 mM Tris.HCl (pH 7.5) were annealed by heating to

90°C for 2 min. and cooling 15 min at 24°C (Fig. 31). Primer extension at 24°C was initiated by addition of 100µL containing 1 mM in all four deoxynucleotide triphosphates, and 20µl Klenow fragment (5 units/l). The extension reaction was stopped every 15 seconds over ten min by addition of 10µl 0.25 M EDTA (pH 8) to 50µl aliquots of the reaction mixture. Samples were pooled, phenol chlorophorm extracted and DNA was precipitated twice by addition of 2.5 vol 100% ethanol, and washed twice with 70% ethanol. The pellet was dried, and redissolved in 0.4 ml 1 mM EDTA, 10 mM Tris (pH 8).

Misincorporation of α-thiodeoxynucleotides onto the 3' ends of the pool of randomly terminated template was carried out by incubating four 0.2 ml solutions each containing one-fourth of the randomly terminated template mixture (~20µg), 0.25 mM of a given α-thiodeoxynucleotide triphosphate, 100 units AMV polymerase, 50 mM KCL, 10 mM $MgCl_2$, 0.4 mM dithiothreitol, and 50 mM Tris (pH 8.3) (Champoux, J.J. (1984) Genetics, 2, 454-464). After incubation at 37°C for 90 minutes, misincorporation reactions were sealed by incubation for five minutes at 37°C with 50 mM all four deoxynucleotide triphosphates (pH 8), and 50 units AMV polymerase. Reactions were stopped by addition of 25 mM EDTA (final), and heated at 68°C for ten min to inactivate AMV polymerase. After ethanol precipitation and resuspension, synthesis of closed circular heteroduplexes was carried out for two days at 14°C under the same conditions used for the timed extension reactions above, except the reactions also contained 1000 units T4 DNA ligase, 0.5 mM ATP and 1 mM β-mercaptoethanol. Simultaneous restriction of each heteroduplex pool with KpnI, BamHI, and EcoRI confirmed that the

extension reactions were nearly quantitative. Heteroduplex DNA in each reaction mixture was methylated by incubation with 80μM S-adenosylmethionine and 150 units dam methylase for 1 hour at 37°C. Methylation reactions were stopped by heating at 68°C for 15 min.

One-half of each of the four methylated heteroduplex reactions were transformed into 2.5 ml competent E. coli JM101 (Messing, J. (1979) Recombinant DNA Tech. Bull., 2, 43-48). The number of independent transformants from each of the four transformations ranged from 0.4-2.0 x 10^5. After growing out phage pools, RF DNA from each of the four transformations was isolated and purified by centrifugation through CsCl density gradients. Approximately 2μg of RF DNA from each of the four pools was digested with EcoRI, BamHI and AvaI. The 1.5 kb EcoRI-BamHI fragment (i.e., AvaI resistant) was purified on low gel temperature agarose and ligated into the 5.5 kb EcoRI-BamHI vector fragment of pB0180. The total number of independent transformants from each α-thiodeoxynucleotide misincorporation plasmid library ranged from 1.2-2.4 x 10^4. The pool of plasmids from each of the four transformations was grown out in 200 ml LB media containing 12.5μg/ml cmp and plasmid DNA was purified by centrifugation through CsCl density gradients.

C. Expression and Screening of Subtilisin Point Mutants

Plasmid DNA from each of the four misincorporation pools was transformed (Anagnostopoulos, C., et al. (1967), J. Bacteriol., 81, 741-746) into BG2036. For each transformation, 5μg of DNA produced approximately

2.5×10^5 independent BG2036 transformants, and liquid culture aliquots from the four libraries were stored in 10% glycerol at 70°C. Thawed aliquots of frozen cultures were plated on LB/5μg/ml cmp/1.6% skim milk plates (Wells, J.A., et al. (1983) Nucleic Acids Res., 11, 7911-7925), and fresh colonies were arrayed onto 96-well microtiter plates containing 150 l per well LB media plus 12.5μg/ml cmp. After 1 h at room temperature, a replica was stamped (using a matched 96 prong stamp) onto a 132 mm BA 85 nitrocellulose filter (Schleicher and Scheull) which was layered on a 140 mm diameter LB/cmp/skim milk plate. Cells were grown about 16 h at 30°C until halos of proteolysis were roughly 5-7 mm in diameter and filters were transferred directly to a freshly prepared agar plate at 37°C containing only 1.6% skim milk and 50 mM sodium phosphate pH 11.5. Filters were incubated on plates for 3-6 h at 37°C to produce halos of about 5 mm for wild-type subtilisin and were discarded. The plates were stained for 10 min at 24°C with Coomassie blue solution (0.25% Coomassie blue (R-250) 25% ethanol) and destained with 25% ethanol, 10% acetic acid for 20 min. Zones of proteolysis appeared as blue halos on a white background on the underside of the plate and were compared to the original growth plate that was similarly stained and destained as a control. Clones were considered positive that produced proportionately larger zones of proteolysis on the high pH plates relative to the original growth plate. Negative clones gave smaller halos under alkaline conditions. Positive and negative clones were restreaked to colony purify and screened again in triplicate to confirm alkaline pH results.

D. Identification and Analysis of Mutant Subtilisins

Plasmid DNA from 5 ml overnight cultures of more alkaline active B.subtilis clones was prepared according to Birnboim and Doly (Birnboim, H.C., et al. (1979) Nucleic Acid Res. 7, 1513) except that incubation with 2 mg/ml lysozyme proceeded for 5 min at 37°C to ensure cell lysis and an additional phenol/$CHCl_3$ extraction was employed to remove contaminants. The 1.5 kb EcoRI-BamHI fragment containing the subtilisin gene was ligated into M13mp11 and template DNA was prepared for DNA sequencing (Messing, J., et al. (1982) Gene, 19 269-276). Three DNA sequencing primers ending at codon 26, +95, and +155 were synthesized to match the subtilisin coding sequence. For preliminary sequence identification a single track of DNA sequence, corresponding to the dNTPaS misincorporation library from which the mutant came, was applied over the entire mature protein coding sequence (i.e., a single dideoxyguanosine sequence track was applied to identify a mutant from the dGTPas library). A complete four track of DNA sequence was performed 200 bp over the site of mutagenesis to confirm and identify the mutant sequence (Sanger, F., et al., (1980) J. Mol. Biol., 143, 161-178). Confirmed positive and negative bacilli clones were cultured in LB media containing 12.5µg/mL cmp and purified from culture supernatants as previously described (Estell, D.A., et al. (1985) J. Biol. Chem., 260, 6518-6521). Enzymes were greater than 98% pure as analyzed by SDS-polyacrylamide gel electrophoresis (Laemmli, U.K. (1970), Nature, 227, 680-685), and protein concentrations were calculated from the absorbance at 280 nm, $\epsilon_{280}^{0.1\%} = 1.17$ (Maturbara, H., et al. (1965), J. Biol. Chem, 240, 1125-1130).

Enzyme activity was measured with 200μg/mL succinyl-L-AlaL-AlaL-ProL-Phep-nitroanilide (Sigma) in 0.1M Tris pH 8.6 or 0.1 M CAPS pH 10.8 at 25°C. Specific activity (μ moles product/min-mg) was calculated from the change in absorbance at 410 nm from production of p-nitroaniline with time per mg of enzyme (E410 = 8,480 M-1cm-1; Del Mar, E.G., et al. (1979), Anal. Biochem., 99, 316-320). Alkaline autolytic stability studies were performed on purified enzymes (200μg/mL) in 0.1 M potassium phosphate (pH 12.0) at 37°C. At various times aliquots were assayed for residual enzyme activity (Wells, J.A., et al. (1986) J. Biol. Chem., 261, 6564-6570).

E. Results

1. Optimization and analysis of mutagenesis frequency

A set of primer-template molecules that were randomly 3'-terminated over the subtilisin gene (Fig. 31) was produced by variable extension from a fixed 5'-primer (The primer mutated a unique AvaI site at codon 11 in the subtilisin gene). This was achieved by stopping polymerase reactions with EDTA after various times of extension. The extent and distribution of duplex formation over the 1 kb subtilisin gene fragment was assessed by multiple restriction digestion (not shown). For example, production of new HinfI fragments identified when polymerase extension had proceeded past Ile110, Leu233, and Asp259 in the subtilisin gene.

Misincorporation of each dNTPαs at randomly terminated 3' ends by AMV reverse transcriptase (Zakour, R.A., et al. (1982), Nature, 295, 708-710; Zakour, R.A., et al. (1984), Nucleic Acids Res., 12, 6615-6628) used

conditions previously described (Champoux, J.J., (1984), Genetics, 2, 454-464). The efficiency of each misincorporation reaction was estimated to be greater than 80% by the addition of each dNTPαs to the AvaI restriction primer, and analysis by polyacrylamide gel electrophoresis. Misincorporations were sealed by polymerization with all four dNTP's and closed circular DNA was produced by reaction with DNA ligase.

Several manipulations were employed to maximize the yield of the mutant sequences in the heteroduplex. These included the use of a deoxyuridine containing template (Kunkel, T.A. (1985), Proc. Natl. Acad. Sci. USA, 82 488-492; Pukkila, P.J. et al. (1983), Genetics, 104, 571-582), in vitro methylation of the mutagenic strand (Kramer, W. et al. (1982) Nucleic Acids Res., 10 6475-6485), and the use of AvaI restriction-selection against the wild-type template strand which contained a unique AvaI site. The separate contribution of each of these enrichment procedures to the final mutagenesis frequency was not determined, except that prior to AvaI restriction-selection roughly one-third of the segregated clones in each of the four pools still retained a wild-type AvaI site within the subtilisin gene. After AvaI restriction-selection greater than 98% of the plasmids lacked the wild-type AvaI site.

The 1.5 kb EcoRI-BamHI subtilisin gene fragment that was resistant to AvaI restriction digestion, from each of the four CsCl purified M13 RF pools was isolated on low melting agarose. The fragment was ligated in situ from the agarose with a similarly cut E. coli-B. subtilis shuttle vector, pB0180, and transformed directly into E coli LE392. Such direct ligation and transformation of DNA isolated from agarose avoided

loses and allowed large numbers of recombinants to be obtained (>100,000 per µg equivalent of input M13 pool).

The frequency of mutagenesis for each of the four dNTPαs misincorporation reactions was estimated from the frequency that unique restriction sites were eliminated (Table XX). The unique restriction sites chosen for this analysis, ClaI, PvuII, and KpnI, were distributed over the subtilisin gene starting at codons 35, 104, and 166, respectively. As a control, the mutagenesis frequency was determined at the PstI site located in the β lactamase gene which was outside the window of mutagenesis. Because the absolute mutagenesis frequency was close to the percentage of undigested plasmid DNA, two rounds of restriction-selection were necessary to reduce the background of surviving uncut wild-type plasmid DNA below the mutant plasmid (Table XX). The background of surviving plasmid from wild-type DNA probably represents the sum total of spontaneous mutations, uncut wild-type plasmid, plus the efficiency with which linear DNA can transform E. coli. Subtracting the frequency for unmutagenized DNA (background) from the frequency for mutant DNA, and normalizing for the window of mutagenesis sampled by a given restriction analysis (4-6 bp) provides an estimate of the mutagenesis efficiency over the entire coding sequence (~1000 bp).

TABLE XX

α-thiol dNTP misincorporated[b]	Restriction Site Selection	% resistant clones[c] 1st round	2nd round	Total	% resistant clones over Background[d]	% mutants per 1000bp[e]
None	PstI	0.32	0.7	0.002	0	-
G	PstI	0.33	1.0	0.003	0.001	0.2
T	PstI	0.32	<0.5	<0.002	0	0
C	PstI	0.43	3.0	0.013	0.011	3
None	ClaI	0.28	5	0.014	0	-
G	ClaI	2.26	85	1.92	1.91	380
T	ClaI	0.48	31	0.15	0.14	35
C	ClaI	0.55	15	0.08	0.066	17
None	PvuII	0.08	29	0.023	0	-
G	PvuII	0.41	90	0.37	0.35	88
T	PvuII	0.10	67	0.067	0.044	9
C	PvuII	0.76	53	0.40	0.38	95
None	KpnI	0.41	3	0.012	0	-
G	KpnI	0.98	35	0.34	0.33	83
T	KpnI	0.36	15	0.054	0.042	8
C	KpnI	1.47	26	0.38	0.37	93

(a) Mutagenesis frequency is estimated from the frequency for obtaining mutations that alter unique restriction sites within the mutagenized subtilisin gene (i.e., ClaI, PvuII, or KpnI) compared to mutation frequencies of the PstI site, that is outside the window of mutagenesis.

(b) Plasmid DNA was from wild-type (none) or mutagenized by dNTPas misincorporation as described.

(c) Percentage of resistant clones was calculated from the fraction of clones obtained after three fold or greater over-digestion of the plasmid with the indicated restriction enzyme compared to a

non-digested control. Restriction-resistant plasmid DNA from the first round was subjected to a second round of restriction-selection. The total represents the product of the fractions of resistant clones obtained from both rounds of selection and gives percentage of restriction-site mutant clones in the original starting pool. Frequencies were derived from counting at least 20 colonies and usually greater than 100.

(d) Percent resistant clones was calculated by subtracting the percentage of restriction-resistant clones obtained for wild-type DNA (i.e., none) from that obtained for mutant DNA.

(e) This extrapolates from the frequency of mutation over each restriction site to the entire subtilisin gene (~1 kb). This has been normalized to the number of possible bases (4-6 bp) within each restriction site that can be mutagenized by a given misincorporation event.

From this analysis, the average percentage of subtilisin genes containing mutations that result from dGTPαs, dCTPαs, or dTTPαs misincorporation was estimated to be 90, 70, and 20 percent, respectively. These high mutagenesis frequencies were generally quite variable depending upon the dNTPαs and misincorporation efficiencies at this site. Misincorporation efficiency has been reported to be both dependent on the kind of mismatch, and the context of primer (Champoux, J.J., (1984); Skinner, J.A., et al. (1986) Nucleic Acids Res., 14, 6945-6964). Biased misincorporation efficiency of dGTPαs and dCTPαs over dTTPαs has been previously observed (Shortle, D., et al. (1985), Genetics, 110, 539-555). Unlike the dGTPαs, dCTPαs, and dTTPαs libraries the efficiency of mutagenesis for the dATPαs

misincorporation library could not be accurately assessed because 90% of the restriction-resistant plasmids analyzed simply lacked the subtilisin gene insert. This problem probably arose from self-ligation of the vector when the dATPαs mutagenized subtilisin gene was subcloned from M13 into pB0180. Correcting for the vector background, we estimate the mutagenesis frequency around 20 percent in the dATPαs misincorporation library. In a separate experiment (not shown), the mutagenesis efficiencies for dGTPαs and dTTPαs misincorporation were estimated to be around 50 and 30 percent, respectively, based on the frequency of reversion of an inactivating mutation at codon 169.

The location and identity of each mutation was determined by a single track of DNA sequencing corresponding to the misincorporated αthiodeoxynucleotide over the entire gene followed by a complete four track of DNA sequencing focused over the site of mutation. Of 14 mutants identified, the distribution was similar to that reported by Shortle and Lin (1985) except we did not observe nucleotide insertion or deletion mutations. The proportion of AG mutations was highest in the G misincorporation library, and some unexpected point mutations appeared in the dTTPαs and dCTPαs libraries.

2. Screening and Identification of Alkaline Stability Mutants of Subtilisin

It is possible to screen colonies producing subtilisin by halos of casein digestion (Wells, J.A. et al. (1983) Nucleic Acids Res., 11, 7911-7925). However, two problems were posed by screening colonies under high alkaline conditions (>pH 11). First, B. subtilis

will not grow at high pH, and we have been unable to transform an alkylophilic strain of bacillus. This problem was overcome by adopting a replica plating strategy in which colonies were grown on filters at neutral pH to produce subtilisin and filters subsequently transferred to casein plates at pH 11.5 to assay subtilisin activity. However, at pH 11.5 the casein micells no longer formed a turbid background and thus prevented a clear observation of proteolysis halos. The problem was overcome by briefly staining the plate with Coomassie blue to amplify proteolysis zones and acidifying the plates to develop casein micell turbidity. By comparison of the halo size produced on the reference growth plate (pH 7) to the high pH plate (pH 11.5), it was possible to identify mutant subtilisins that had increased (positives) or decreased (negatives) stability under alkaline conditions.

Roughly 1000 colonies were screened from each of the four misincorporation libraries. The percentage of colonies showing a differential loss of activity at pH 11.5 versus pH 7 represented 1.4, 1.8, 1.4, and 0.6% of the total colonies screened from the thiol dGTPαs, dATPαs, dTTPαs, and dCTPαs libraries, respectively. Several of these negative clones were sequenced and all were found to contain a single base change as expected from the misincorporation library from which they came. Negative mutants included A36, E170 and V50. Two positive mutants were identified as V107 and R213. The ratio of negatives to positives was roughly 50:1.

3. Stability and Activity of Subtilisin Mutants at Alkaline pH

Subtilisin mutants were purified and their autolytic stabilities were measured by the time course of inactivation at pH 12.0 (Figs. 32 and 33). Positive mutants identified from the screen (i.e., V107 and R213) were more resistant to alkaline induced autolytic inactivation compared to wild-type; negative mutants (i.e., E170 and V50) were less resistant. We had advantageously produced another mutant at position 50 (P50) by site-directed mutagenesis. This mutant was more stable than wild-type enzyme to alkaline autolytic inactivation (Fig. 33) At the termination of the autolysis study, SDS-PAGE analysis confirmed that each subtilisin variant had autolyzed to an extent consistent with the remaining enzyme activity.

The stabilizing effects of V107, R213, and F50 are cumulative. See Table XXI. The double mutant, V107/R213 (made by subcloning the 920 bp EcoRI-KpnI fragment of pB0180V107 into the 6.6 kb EcoRI-KpnI fragment of pB0180R213), is more stable than either single mutant. The triple mutant, F50/V107/R213 (made by subcloning the 735 bp EcoRI-PvuII fragment of pF50 (Example 2) into the 6.8 kb EcoRI-PvuII fragment of pB0180/V107, is more stable than the double mutant V107/R213 or F50. The inactivation curves show a biphasic character that becomes more pronounced the more stable the mutant analyzed. This may result from some destablizing chemical modification(s) (eg., deamidation) during the autolysis study and/or reduced stabilization caused by complete digestion of larger autolysis peptides. These alkaline autolysis studies have been repeated on separately purified enzyme batches with essentially the same results. Rates of autolysis should depend both on the conformational

F. Random Cassette Mutagenesis of Residues 197 through 228

Plasmid pΔ222 (Wells, et al. (1985) Gene 34, 315-323) was digested with PstI and BamHI and the 0.4 kb PstI/BamHI fragment (fragment 1, see Fig. 34) purified from a polyacrylamide gel by electroelution.

The 1.5 kb EcoRI/BamHI fragment from pS4.5 was cloned into M13mp9. Site directed mutagenesis was performed to create the A197 mutant and simultaneously insert a silent SstI site over codons 195-196. The mutant EcoRI/BamHI fragment was cloned back into pBS42. The pA197 plasmid was digested with BamHI and SstI and the 5.3 kb BamHI/SstI fragment (fragment 2) was purified from low melting agarose.

Complimentary oligonucleotides were synthesized to span the region from SstI (codons 195-196) to PstI (codons 228-230). These oligodeoxynucleotides were designed to (1) restore codon 197 to the wild type, (2) re-create a silent KpnI site present in pΔ222 at codons 219-220, (3) create a silent SmaI site over codons 210-211, and (4) eliminate the PstI site over codons 228-230 (see Fig. 35). Oligodeoxynucleotides were synthesized with 2% contaminating nucleotides at each cycle of synthesis, e.g., dATP reagent was spiked with 2% dCTP, 2% dGTP, and 2% dTTP. For 97-mers, this 2% poisoning should give the following percentages of non-mutant, single mutants and double or higher mutants per strand with two or more misincorporations per complimentary strand: 14% non-mutant, 28% single mutant, and 57% with ≥2 mutations, according to the general formula

$$f = \frac{\mu^n}{n!} e^{-\mu} .$$

where μ is the average number of mutations and n is a number class of mutations and f is the fraction of the total having that number of mutations. Complimentary oligodeoxynucleotide pools were phosphorylated and annealed (fragment 3) and then ligated at 2-fold molar excess over fragments 1 and 2 in a three-way ligation.

E. coli MM294 was transformed with the ligation reaction, the transformation pool grown up over night and the pooled plasmid DNA was isolated. This pool represented 3.4×10^4 independent transformants. This plasmid pool was digested with PstI and then used to retransform E. coli. A second plasmid pool was prepared and used to transform B. subtilis (BG2036). Approximately 40% of the BG2036 transformants actively expressed subtilisin as judged by halo-clearing on casein plates. Several of the non-expressing transformants were sequenced and found to have insertions or deletions in the synthetic cassettes. Expressing BG2036 mutants were arrayed in microtiter dishes with 150μl of LB/12.5μg/mL chloramphenicol (cmp) per well, incubated at 37°C for 3-4 hours and then stamped in duplicate onto nitrocellulose filters laid on LB 1.5% skim milk/5μg/mL cmp plates and incubated overnight at 33°C (until halos were approximately 4-8 mm in diameter). Filters were then lifted to stacks of filter paper saturated with 1 x Tide commercial grade detergent, 50 mM Na_2CO_3, pH 11.5 and incubated at 65°C for 90 min. Overnight growth plates were Commassie stained and destained to establish basal levels of expression. After this treatment, filters were returned to pH7/skim milk/20μg/mL tetracycline plates and incubated at 37°C for 4 hours to overnight.

Mutants identified by the high pH stability screen to be more alkaline stable were purified and analyzed for autolytic stability at high pH or high temperature. The double mutant C204/R213 was more stable than wild type at either high pH or high temperature (Table XXII).

This mutant was dissected into single mutant parents (C204 and R213) by cutting at the unique SmaI restriction site (Fig. 35) and either ligating wild type sequence 3' to the SmaI site to create the single C204 mutant or ligating wild type sequence 5' to the SmaI site to create the single R213 mutant. Of the two single parents, C204 was nearly as alkaline stable as the parent double mutant (C04/R213) and slightly more thermally stable. See Table XXII. The R213 mutant was only slightly more stable than wild type under both conditions (not shown).

Another mutant identified from the screen of the 197 to 228 random cassette mutagenesis was R204. This mutant was more stable than wild type at both high pH and high temperature but less stable than C204.

TABLE XXII

Stability of subtilisin variants

Purified enzymes (200μg/mL) were incubated in 0.1M phosphate, pH 12 at 30°C for alkaline autolysis, or in 2mM $CaCl_2$, 50mM MOPS, pH 7.0 at 62°C for thermal autolysis. At various times samples were assayed for residual enzyme activity. Inactivations were roughly pseudo-first order, and t 1/2 gives the time it took to reach 50% of the starting activity in two separate experiments.

Subtilisin variant	t 1/2 (alkaline autolysis) Exp. #1	Exp. #2	t 1/2 (thermal autolysis) Exp. #1	Exp. #2
wild type	30	25	20	23
F50/V107/R213	49	41	18	23
R204	35	32	24	27
C204	43	46	38	40
C204/R213	50	52	32	36
L204/R213	32	30	20	21

G. Random Mutagenesis at Codon 204

Based on the above results, codon 204 was targeted for random mutagenesis. Mutagenic DNA cassettes (for codon at 204) all contained a fixed R213 mutation which was found to slightly augment the stability of the C204 mutant.

Plasmid DNA encoding the subtilisin mutant C204/R213 was digested with SstI and EcoRI and a 1.0 kb EcoRI/SstI fragment was isolated by electro-elution from polyacrylamide gel (fragment 1, see Fig. 35).

C204/R213 was also digested with SmaI and EcoRI and the large 4.7 kb fragment, including vector sequences and the 3' portion of coding region, was isolated from low melting agarose (fragment 2, see Fig. 36).

Fragments 1 and 2 were combined in four separate three-way ligations with heterophosphorylated fragments 3 (see Figs. 36 and 37). This heterophosphorylation of synthetic duplexes should preferentially drive the phosphorylated strand into the plasmid ligation product. Four plasmid pools, corresponding to the four ligations, were restricted with SmaI in order to linearize any single cut C204/R213 present from fragment 2 isolation, thus reducing the background of C204/R213. E. coli was then re-transformed with SmaI-restricted plasmid pools to yield a second set of plasmid pools which are essentially free of C204/R213 and any non-segregated heterduplex material.

These second enriched plasmid pools were then used to transform B. subtilis (BG2036) and the resulting four mutant pools were screened for clones expressing subtilisin resistant to high pH/temperature inactivation. Mutants found positive by such a screen were further characterized and identified by sequencing.

The mutant L204/R213 was found to be slightly more stable than the wild type subtilisin. See Table XXII.

Having described the preferred embodiments of the present invention, it will appear to those ordinarily skilled in the art that various modifications may be made to the disclosed embodiments, and that such modifications are intended to be within the scope of the present invention.

CLAIMS;

1. A carbonyl hydrolase mutant having at least one property which is substantially different from the same property of a precursor carbonyl hydrolase from which the amino acid sequence of said carbonyl hydrolase mutant is derived, said property being selected from the group consisting of thermal stability and alkaline stability wherein said precursor carbonyl hydrolase is selected from the group consisting of naturally occurring carbonyl hydrolases and recombinant carbonyl hydrolases and said carbonyl hydrolase mutant amino acid sequence is derived by a method selected from the group consisting of the substitution, deletion and insertion of at least one amino acid in said amino acid sequence of said precursor carbonyl hydrolase.

2. A carbonyl hydrolase mutant having at least one property which is substantially different from the same property of a precursor carbonyl hydrolase from which the amino acid sequence of said carbonyl hydrolase mutant is derived, said property being selected from the group consisting of oxidative stability, substrate specificity, catalytic activity, thermal stability, alkaline stability and pH activity profile wherein said precursor carbonyl hydrolase is selected from the group consisting of naturally occurring carbonyl hydrolases and recombinant carbonyl hydrolases and said carbonyl hydrolase mutant amino acid sequence is derived by a method selected from the group consisting of deletion and insertion of at least one amino acid in said amino acid sequence of said precursor carbonyl hydrolase and substitution of more than one amino acid residue of said amino acid sequence of said precursor carbonyl hydrolase.

3. A carbonyl hydrolase mutant derived by the replacement of at least one amino acid residue of a precursor carbonyl hydrolase with a different amino acid, said one amino acid residue being selected from the group of amino acid residues of Bacillus amyloliquefaciens subtilisin consisting of Tyr21, Thr22, Ser24, Ser33, Asp36, Ala45, Gly46, Ala48, Ser49, Met50, Asn77, Ser87, Lys94, Val95, Leu96, Tyr104, Ile107, Gly110, Met124, Asn155, Glu156, Lys170, Tyr171, Pro172, Phe189, Asp197, Met199, Ser204, Lys213, Tyr217, Ser221, His67, Leu126, Leu135, Gly97, Asp99, Ser101, Gly102, Glu103, Leu126, Gly127, Gly128, Pro129, Tyr214, Gly215, and equivalent amino acid residues in other precursor carbonyl hydrolases.

4. A carbonyl hydrolase mutant having an amino acid sequence derived from the amino acid sequence of a precursor carbonyl hydrolase by the substituion of a differnt amino acid for more than one amino acid residue of said amino acid sequence of said precursor carbonyl hydrolase, said amino acid residues being selected from the group of amino acid residues of Bacillus amyloliquefaciens subtilisin consisting of Tyr21, Thr22, Ser24, Asp32, Ser33, Asp36, Ala45, Gly46, Ala48, Ser49, Met50, Asn77, Ser87, Lys94, Val95, Leu96, Tyr104, Ile107, Gly110, Met124, Ala152, Asn-155, Glu156, Gly166, Gly169, Lys170, Tyr171, Pro172, Phe189, Asp197, Met199, Ser204, Lys213, Tyr217, Ser221, Met222, His67, Leu126, Leu135, Gly97, Asp99, Ser101, Gly102, Glu103, Leu126, Gly127, Gly128, Pro129, Tyr214, Gly215, and equivalent amino acid residues in other precursor carbonyl hydrolases.

5. The mutant of Claim 4 wherein said combinations are selected from the group consisting of Thr22/Ser87, Ser24/Ser87, Ala45/Ala48, Ser49/Lys94, Ser49/Val95, Met50/Val95, Met50/Gly110, Met50/Met124, Met50/Met222, Met124/Met222, Glu156/Gly166, Glu156/Gly169, Gly166/Met222, Gly169/Met222, Tyr21/Thr22, Met50/Met124/Met222, Tyr21/Thr22/Ser87, Met50/Glu156/Gly166/Tyr217, Met50/Glu156/Tyr217, Glu156/Gly169/Tyr217, Ile170/Lys213, Ser204/Lys213, Met50/Ile107/Lys213 and Ser24/Met50/Ile107/Glu156/Gly166/Gly169/Ser204/Lys213/Gly215/Tyr217.

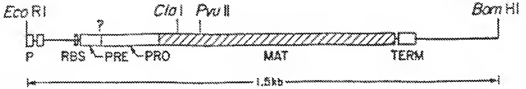
6. A carbonyl hydrolase mutant derived by the replacement of at least one amino acid residue of a precursor carbonyl hydrolase with a different amino acid, said one amino acid residue being selected from the group of amino acid residues of of Bacillus amyloliquefaciens subtilisin consisting of Tyr21, Thr22, Ser24, Asp32, Ser33, Asp36, Ala45, Gly46, Ala48, Ser49, Met50, Asn77, Ser87, Lys94, Val95, Leu96, Tyr104, Ile107, Gly110, Met124, Ala152, Asn-155, Glu156, Gly166, Gly169, Lys170, Tyr171, Pro172, Phe189, Asp197, Met199, Ser204, Lys213, Tyr217, Ser221, Met222, His67, Leu126, Leu135, Gly97, Asp99, Ser101, Gly102, Glu103, Leu126, Gly127, Gly128, Pro129, Tyr214, Gly215, and equivalent amino acid residues in other precursor carbonyl hydrolases, wherein said at least one amino acid residue of said precursor carbonyl hydrolase is replaced with the amino acid residues listed in TABLE I and TABLE II herein.

7. The mutant of Claim 6 wherein the amino acid replacing said at least one amino acid residue in said precursor carbonyl hydrolase is selectd from the replacement amino acids listed in TABLE I herein.

8. Mutant DNA sequence encoding the mutant of claims 1 through 7.

9. Expression vector containing the mutant DNA sequence of claim 8.

10. Host cell transformed with the expression vector of Claim 9.




```
 (5)                                  (3)                                 (4)
            P                                                                                  RBS         -107
                                                                                                           fMet
1 GGTCTACTAAAATATTATTCCATACTATACAATTAATACACAGAATAATCTGTCTATTGGTTATTCTGCAAATGAAAAAAAGGAGAGGATAAAGA GTG

                                  -100             PRE                     -90
   Arg Gly Lys Lys Val Trp Ile Ser Leu Leu Phe Ala Leu Ala Leu Ile Phe Thr Met Ala Phe Gly Ser Thr Ser
99 AGA GGC AAA AAA GTA TGG ATC AGT TTG CTG TTT GCT TTA GCG TTA ATC TTT ACG ATG GCG TTC GGC AGC ACA TCC

    -80                                  -70        PRO                          -60
    Ser Ala Gln Ala Ala Gly Lys Ser Asn Gly Glu Lys Lys Tyr Ile Val Gly Phe Lys Gln Thr Met Ser Thr Met
174 TCT GCC CAG GCG GCA GGG AAA TCA AAC GGG GAA AAG AAA TAT ATT GTC GGG TTT AAA CAG ACA ATG AGC ACG ATG

                            -50                                     -40
    Ser Ala Ala Lys Lys Lys Asp Val Ile Ser Glu Lys Gly Gly Lys Val Gln Lys Gln Phe Lys Tyr Val Asp Ala
249 AGC GCC GCT AAG AAG AAA GAT GTC ATT TCT GAA AAA GGC GGG AAA GTG CAA AAG CAA TTC AAA TAT GTA GAC GCA

        -30                                     -20                                     -10
    Ala Ser Ala Thr Leu Asn Glu Lys Ala Val Lys Glu Leu Lys Lys Asp Pro Ser Val Ala Tyr Val Glu Glu Asp
324 GCT TCA GCT ACA TTA AAC GAA AAA GCT GTA AAA GAA TTG AAA AAA GAC CCG AGC GTC GCT TAC GTT GAA GAA GAT

                         -1 1 ---> MAT                    10
    His Val Ala His Ala Tyr Ala Gln Ser Val Pro Tyr Gly Val Ser Gln Ile Lys Ala Pro Ala Leu His Ser Gln
399 CAC GTA GCA CAT GCG TAC GCG CAG TCC GTG CCT TAC GGC GTA TCA CAA ATT AAA GCC CCT GCT CTG CAC TCT CAA

    20                                      30                                      40
    Gly Tyr Thr Gly Ser Asn Val Lys Val Ala Val Ile Asp Ser Gly Ile Asp Ser Ser His Pro Asp Leu Lys Val
474 GGC TAC ACT GGA TCA AAT GTT AAA GTA GCG GTT ATC GAC AGC GGT ATC GAT TCT TCT CAT CCT GAT TTA AAG GTA

                        50                      Pro Asn             60 Asp
    Ala Gly Gly Ala Ser Met Val Pro Ser Glu Thr Asn Pro Phe Gln Asp Asn Asn Ser His Gly Thr His Val Ala
549 GCA GGC GGA GCC AGC ATG GTT CCT TCT GAA ACA AAT CCT TTC CAA GAC AAC AAC TCT CAC GGA ACT CAC GTT GCC

    70                                      80                          Ser Ala  90
    Gly Thr Val Ala Ala Leu Asn Asn Ser Ile Gly Val Leu Gly Val Ala Pro Ser Ala Ser Leu Tyr Ala Val Lys
624 GGC ACA GTT GCG GCT CTT AAT AAC TCA ATC GGT GTA TTA GGC GTT GCG CCA AGC GCA TCA CTT TAC GCT GTA AAA

                Asn Ala 100                                 110
    Val Leu Gly Ala Asp Gly Ser Gly Gln Tyr Ser Trp Ile Ile Asn Gly Ile Glu Trp Ala Ile Ala Asn Asn Met
699 GTT CTC GGT GCT GAC GGT TCC GGC CAA TAC AGC TGG ATC ATT AAC GGA ATC GAG TGG GCG ATC GCA AAC AAT ATG

    120                                     130                                     140
    Asp Val Ile Asn Met Ser Leu Gly Gly Pro Ser Gly Ser Ala Ala Leu Lys Ala Ala Val Asp Lys Ala Val Ala
774 GAC GTT ATT AAC ATG AGC CTC GGC GGA CCT TCT GGT TCT GCT GCT TTA AAA GCG GCA GTT GAT AAA GCC GTT GCA

                        150                             Ser Thr 160
    Ser Gly Val Val Val Val Ala Ala Ala Gly Asn Glu Gly Thr Ser Gly Ser Ser Ser Thr Val Gly Tyr Pro Gly
849 TCC GGC GTC GTA GTC GTT GCG GCA GCC GGT AAC GAA GGC ACT TCC GGC AGC TCA AGC ACA GTG GGC TAC CCT GGT

    170                                     180                                     190
    Lys Tyr Pro Ser Val Ile Ala Val Gly Ala Val Asp Ser Ser Asn Gln Arg Ala Ser Phe Ser Ser Val Gly Pro
924 AAA TAC CCT TCT GTC ATT GCA GTA GGC GCT GTT GAC AGC AGC AAC CAA AGA GCA TCT TTC TCA AGC GTA GGA CCT

                        200                                     210
    Glu Leu Asp Val Met Ala Pro Gly Val Ser Ile Gln Ser Thr Leu Pro Gly Asn Lys Tyr Gly Ala Tyr Asn Gly
999 GAG CTT GAT GTC ATG GCA CCT GGC GTA TCT ATC CAA AGC ACG CTT CCT GGA AAC AAA TAC GGG GCG TAC AAC GGT

     220                                     230                                     240
     Thr Ser Met Ala Ser Pro His Val Ala Gly Ala Ala Ala Leu Ile Leu Ser Lys His Pro Asn Trp Thr Asn Thr
1074 ACG TCA ATG GCA TCT CCG CAC GTT GCC GGA GCG GCT GCT TTG ATT CTT TCT AAG CAC CCG AAC TGG ACA AAC ACT

                         250 Gln                                260
     Gln Val Arg Ser Ser Leu Glu Asn Thr Thr Thr Lys Leu Gly Asp Ser Phe Tyr Tyr Gly Lys Gly Leu Ile Asn
1149 CAA GTC CGC AGC AGT TTA GAA AAC ACC ACT ACA AAA CTT GGT GAT TCT TTC TAC TAT GGA AAA GGG CTG ATC AAC

     270                 275
     Val Gln Ala Ala Ala Gln OC               TERM
1224 GTA CAG GCG GCA GCT CAG TAA  AACATAAAAAACCGGCCTTGGCCCCGCCGGTTTTTTATTATTTTTCTTCCTCCGCATGTTCAATCCGCTCC

1316 ATAATCGACGGATGGCTCCCTCTGAAAATTTTAACGAGAAACGGCGGGTTGACCCGGCTCAGTCCCGTAACGGCCAAGTCCTGAAACGTCTCAATCGCCG

1416 CTTCCCGGTTTCCGGTCAGCTCAATGCCGTAACGGTCGGCGGCGTTTTCCTGATACCGGGAGACGGCATTCGTAATCGGATC
```

FIG.—1

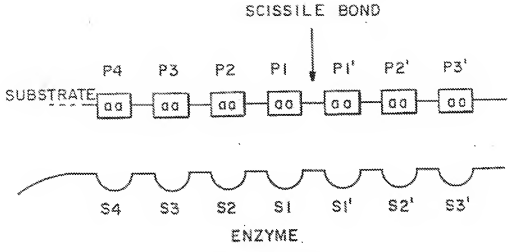

SCISSILE BOND
P4
P3
P2
P1
P1'
P2'
P3'
SUBSTRATE
aa
S4
S3
S2
S1
S1'
S2'
S3'
ENZYME

FIG.—2

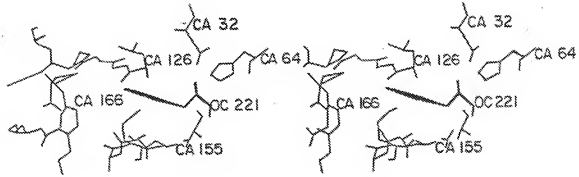

CA 32
CA 126
CA 64
CA 166
OC 221
CA 155
CA 32
CA 126
CA 64
CA 166
OC 221
CA 155

FIG.—3

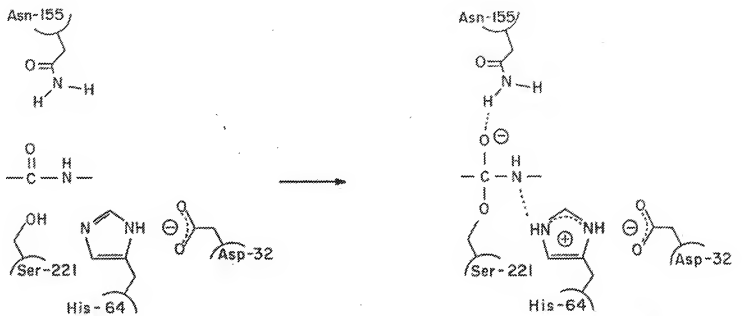


FIG.—4

0251446

Homology of Bacillus proteases

1.Bacillus amyloliquifaciens
2.Bacillus subtilis var.I168
3.Bacillus licheniformis (carlsbergensis)

1									10									20
A	Q	S	V	P	Y	G	V	S	Q	I	K	A	P	A	L	H	S	Q
A	Q	S	V	P	Y	G	I	S	Q	I	K	A	P	A	L	H	S	Q
A	Q	T	V	P	Y	G	I	P	L	I	K	A	D	K	V	Q	A	Q

21									30									40
Y	T	G	S	N	V	K	V	A	V	I	D	S	G	I	D	S	S	H
Y	T	G	S	N	V	K	V	A	V	I	D	S	G	I	D	S	G	H
F	K	G	A	N	V	K	V	A	V	L	D	T	G	I	Q	A	S	H

41									50									60
D	L	K	V	A	G	G	A	S	M	V	P	S	E	T	N	P	F	Q
D	L	N	V	R	G	G	A	S	F	V	P	S	E	T	N	P	Y	Q
D	L	N	V	V	G	G	A	S	F	V	A	G	E	A	Y	N	T	*

61									70									80
N	N	S	H	G	T	H	V	A	G	T	V	A	A	L	N	N	S	I
G	S	S	H	G	T	H	V	A	G	T	I	A	A	L	N	N	S	I
G	N	G	H	G	T	H	V	A	G	T	V	A	A	L	D	N	T	T

81									90									100
V	L	G	V	A	P	S	A	S	L	Y	A	V	K	V	L	G	A	D
V	L	G	V	S	P	S	A	S	L	Y	A	V	K	V	L	D	S	T
V	L	G	V	A	P	S	V	S	L	Y	A	V	K	V	L	N	S	S

101									110									120
S	G	Q	Y	S	W	I	I	N	G	I	E	W	A	I	A	N	N	M
S	G	Q	Y	S	W	I	I	N	G	I	E	W	A	I	S	N	N	M
S	G	S	Y	S	G	I	V	S	G	I	E	W	A	T	T	N	G	M

FIG.—5A-1

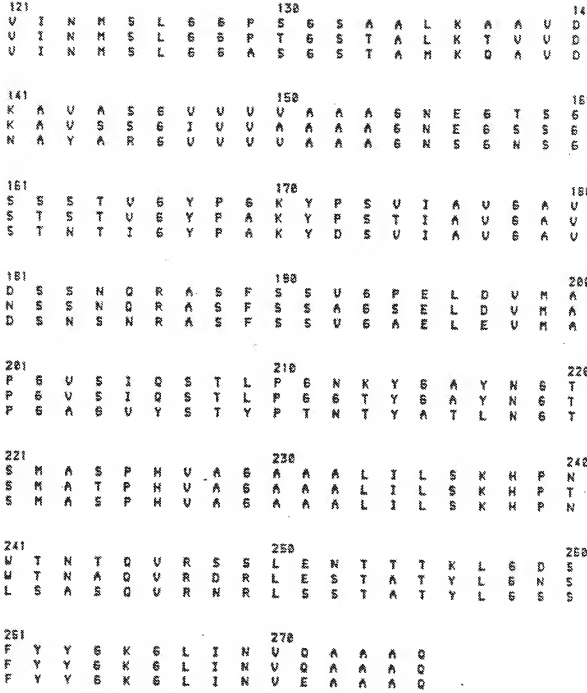


FIG.—5A—2

ALIGNMENT OF B.AMYLOLIQUIFACIENS SUBTILISIN AND THERMITASE
1.B.amyloliquifaciens subtilisin
2.thermitase

1																10			
A	Q	S	V	*	P	Y	*	*	*	*	*	*	G	V	S	Q	I	K	A
Y	T	P	N	D	P	Y	F	S	S	R	Q	Y	G	P	Q	K	I	Q	A

						20										30			
P	A	L	H	S	Q	G	Y	T	G	S	N	V	K	V	A	V	I	D	S
P	Q	A	W	D	I	A	E	*	G	S	G	A	K	I	A	I	V	D	T

						40												50	
G	I	D	S	S	H	P	D	L	*	*	K	V	A	G	G	A	S	M	V
G	V	Q	S	N	H	P	D	L	A	G	K	V	V	G	G	W	D	F	V

								60										70	
P	S	E	T	N	P	F	Q	D	N	N	S	H	G	T	H	V	A	G	T
D	N	D	S	T	P	*	Q	N	G	N	G	H	G	T	H	C	A	G	I

									80										90
V	A	A	L	*	N	N	S	I	G	V	L	G	V	A	P	S	A	S	L
A	A	A	V	T	N	N	S	T	G	I	A	G	T	A	P	K	A	S	I

									100										110
Y	A	V	K	V	L	G	A	D	G	S	G	Q	Y	S	W	I	I	N	G
L	A	V	R	V	L	D	N	S	G	S	G	T	W	T	A	V	A	N	G

									120										130
I	E	W	A	I	A	N	N	M	D	V	I	N	M	S	L	G	G	P	S
I	T	Y	A	A	D	Q	G	A	K	V	I	S	L	S	L	G	G	T	V

									140										150
G	S	A	A	L	K	A	A	V	D	K	A	V	A	S	G	V	V	V	V
G	N	S	G	L	Q	Q	A	V	N	Y	A	W	N	K	G	S	V	V	V

FIG.—5B—I

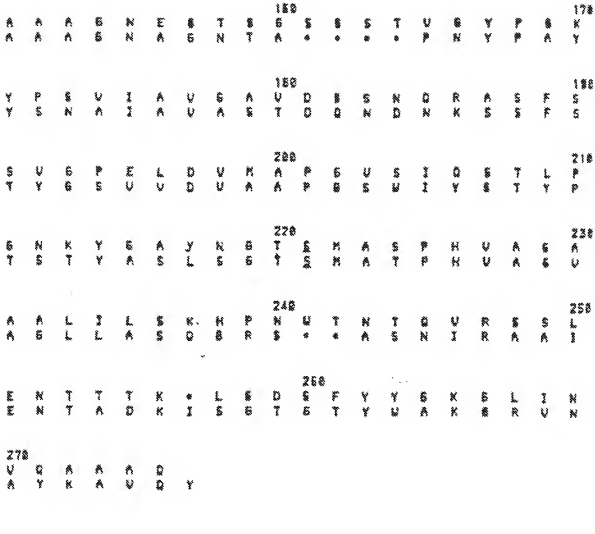


FIG.—5B-2

TOTALLY CONSERVED RESIDUES IN SUBTILISINS

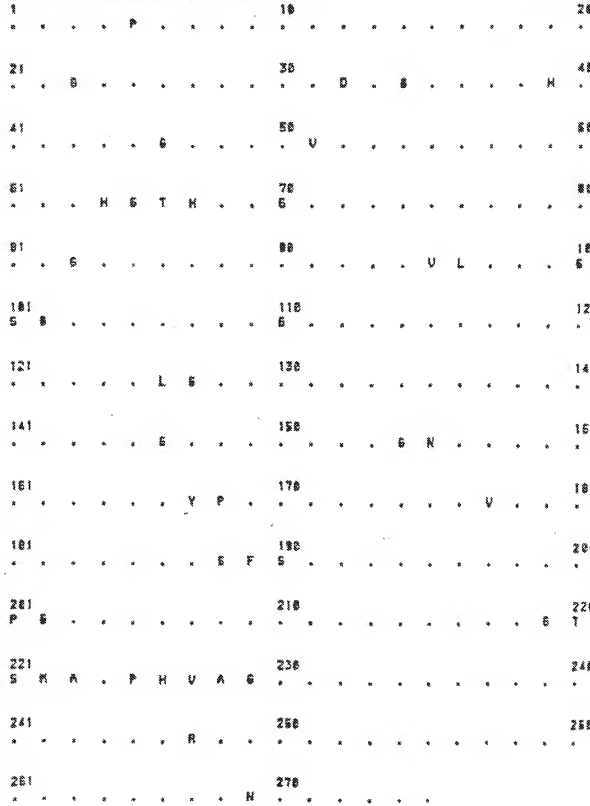


FIG.—5C

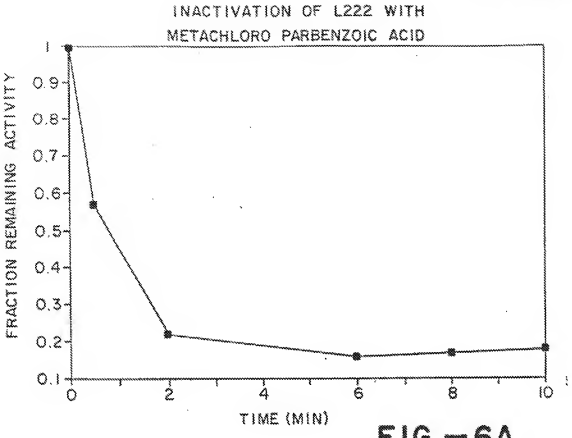

INACTIVATION OF L222 WITH
METACHLORO PARBENZOIC ACID
FRACTION REMAINING ACTIVITY
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
2
4
6
8
10
TIME (MIN)

FIG.—6A

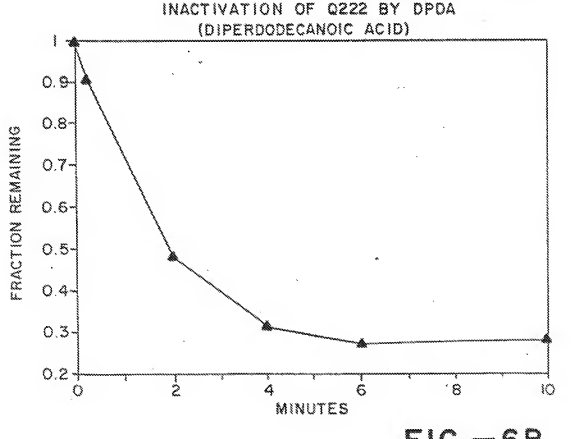

INACTIVATION OF Q222 BY DPDA
(DIPERDODECANOIC ACID)
FRACTION REMAINING
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0
2
4
6
8
10
MINUTES

FIG.—6B

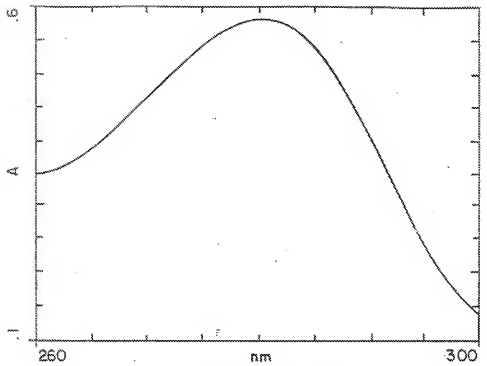


FIG. — 7A

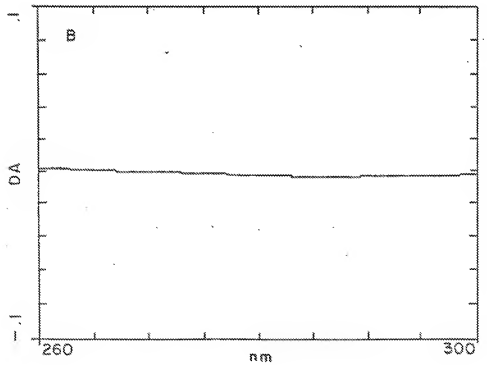


FIG. — 7B

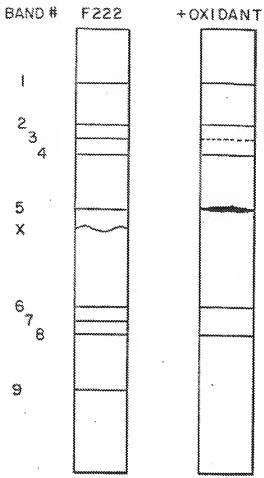

BAND #
F222
+OXIDANT
1
2
3
4
5
X
6
7
8
9

FIG.— 8

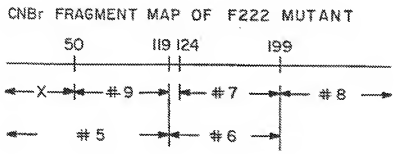

CNBr FRAGMENT MAP OF F222 MUTANT
50
119 124
199
X
#9
#7
#8
#5
#6

FIG.—9

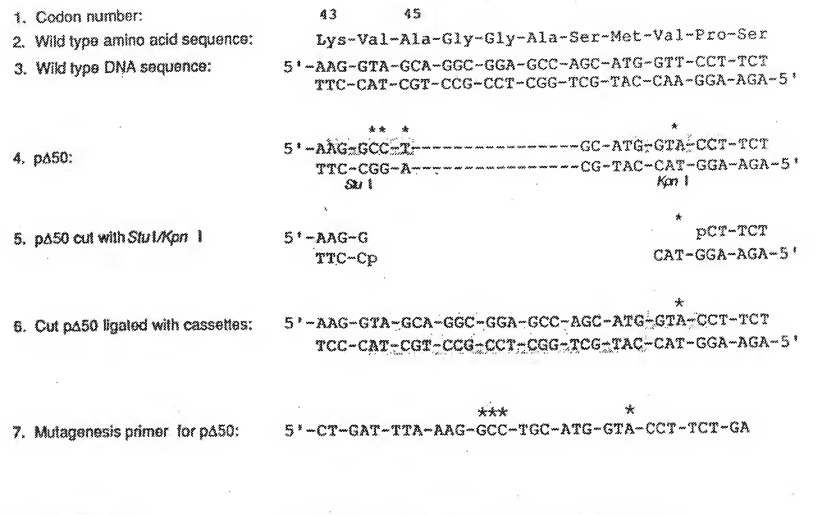


FIG.—10

0251446

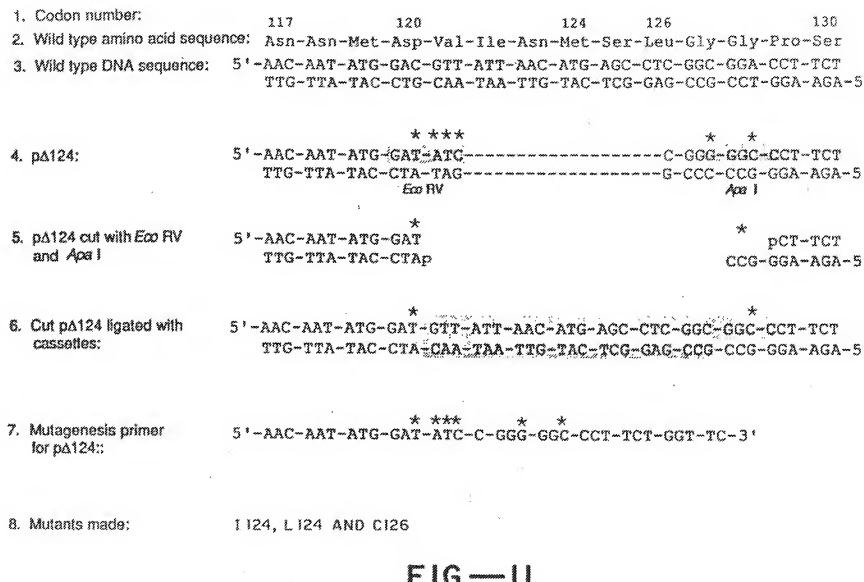


FIG.—11

0251446

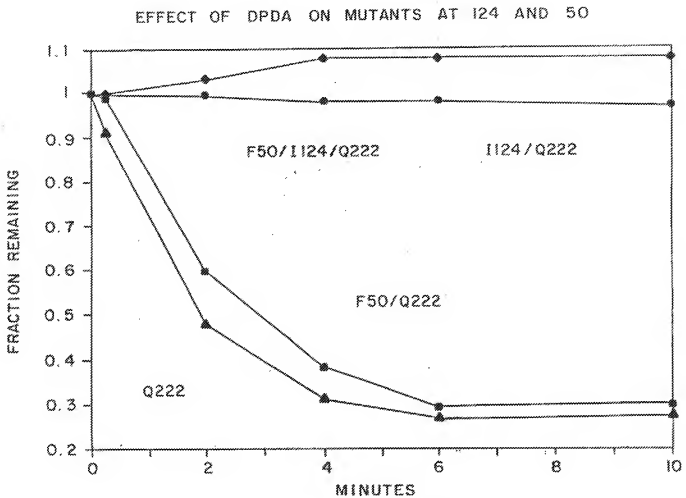
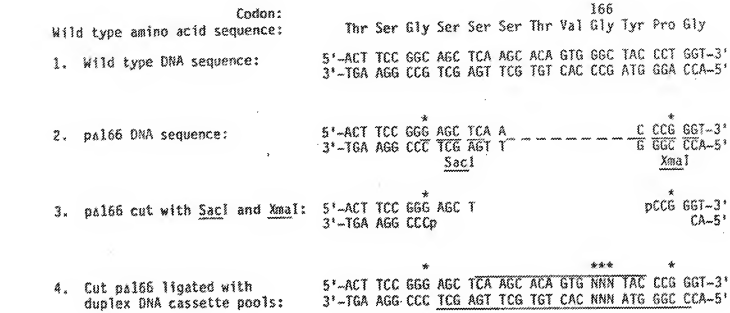

EFFECT OF DPDA ON MUTANTS AT I24 AND 50
FRACTION REMAINING
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0
2
4
6
8
10
MINUTES
F50/I124/Q222
I124/Q222
F50/Q222
Q222

FIG.-12



MUTAGENESIS PRIMER 37MER

5' AA GGC ACT TCC GGG AGC TCA ACC CGG GTA AA TAC CCT 3'

FIG.—13

0251446

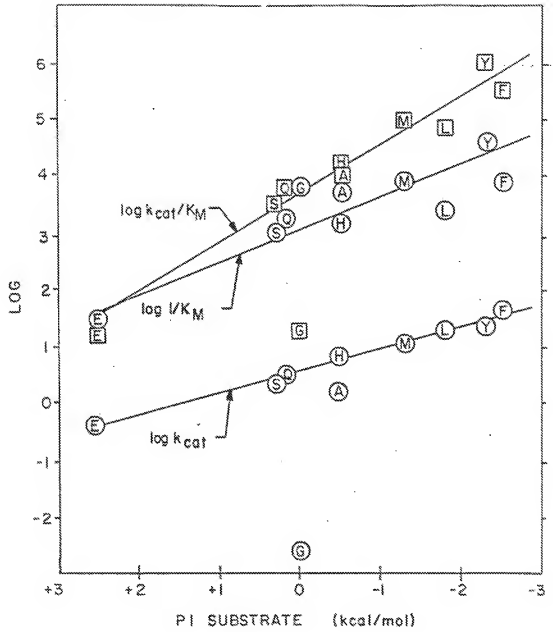

LOG
6
5
4
3
2
1
0
-1
-2
log k_{cat}/K_M
log $1/K_M$
log k_{cat}
+3
+2
+1
0
-1
-2
-3
PI SUBSTRATE (kcal/mol)

FIG. – 14

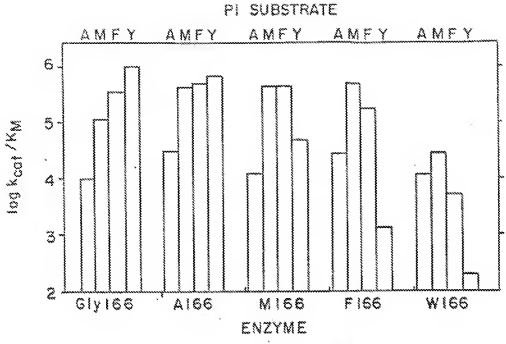


FIG.—15A

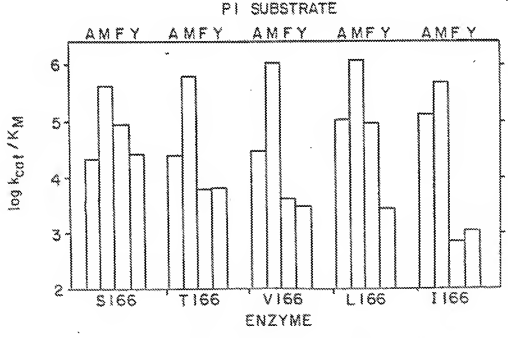


FIG.—15B

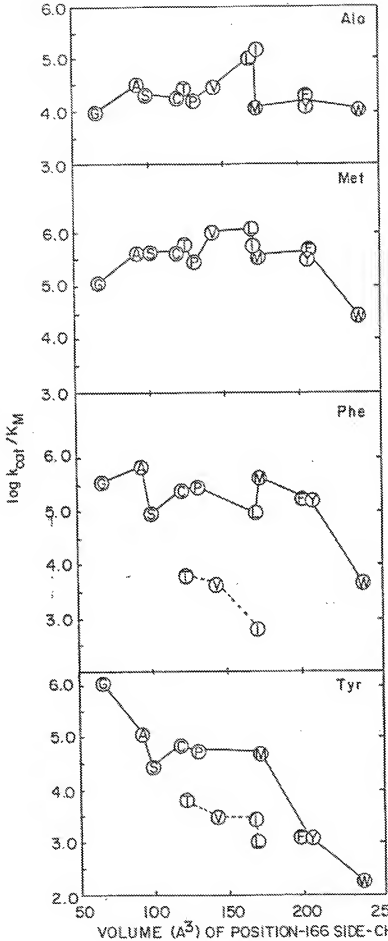

Ala
Met
Phe
Tyr
log k_{cat}/K_M
6.0
5.0
4.0
3.0
2.0
50
100
150
200
250
VOLUME (A^3) OF POSITION-166 SIDE-CHAIN

FIG.-16

$\frac{(k_{cat}/K_M)\ Ile\ 166}{\square \log\ (k_{cat}/K_M)\ Gly\ 166}$
2
1
0
-1
-2
-3
Ala
Val
Met
Leu
His
Phe
Tyr
P1 SUBSTRATE

FIG. – 17

GLY-169 CASSETTE MUTAGENESIS

CODON:		162 169 173
WILD TYPE AMINO ACID SEQUENCE:		SER SER THR VAL GLY TYR PRO GLY LIS TYR PRO SER
1. WILD TYPE DNA SEQUENCE	5'	TCA AGC ACA GTG GGC TAC CCT GGT AAA TAC CCT TCT 3'
	3'	AGT TCG TGT CAC CCG ATG GGA CCA TTT ATG GGA AGA 5'
2. P169 DNA SEQUENCE	5'	TCA AGC ACA GTC GGG TAC CCT-----GA TAT CCT TCT 3'
	3'	AGT TCG TGT CAC CCC ATG GGA CT ATA GGA AGA 5'
		KpnI EcoRV
3. P169 CUT WITH KpnI AND EcoRV:	5'	TAC AGC ACA GTC GGG TAC PAT CCT TCT 3'
	3'	AGT TCG TGT CAC CCp TA GGA AGA 5'
4. CUT P169 LIGATED WITH	5'	TAC AGC ACA GTG GGG TAC CCT NNN AAA TAT CCT TGT 3'
OLIGONUCLEOTIDE POOLS	3'	AGT TCG TGT CAC CCC ATG GGA NNN TTT ATA GGA AGA 5'
MUTAGENESIS PRIMER FOR P169	5'	AAG CAC AGT GGG GTA CCC TGA TAT CCT TCT GTC A 3'

FIG.—18

0251446

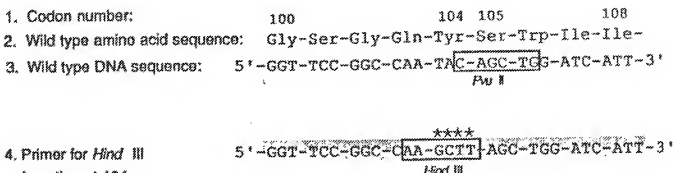


FIG.—19

0251446

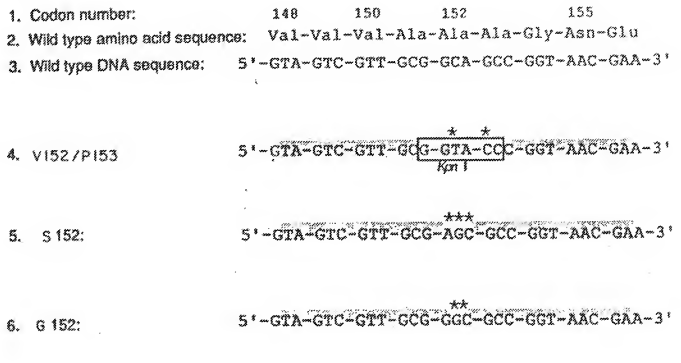


FIG.—20

0251446

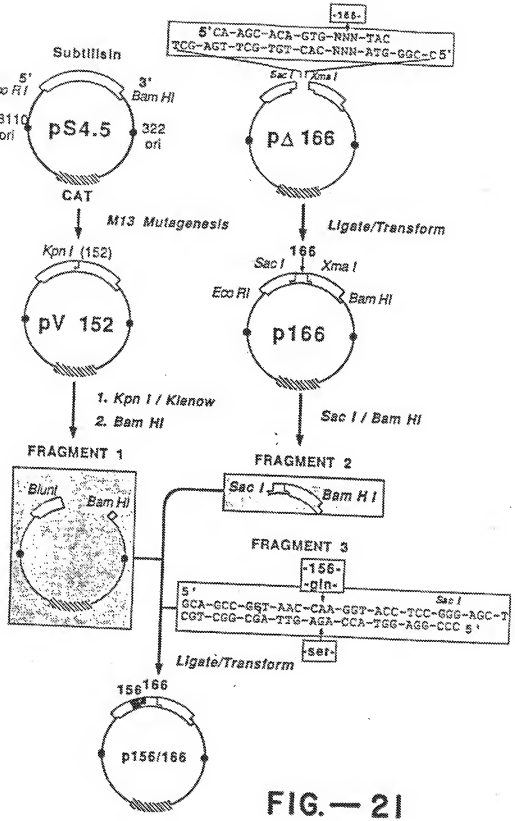

-166-
5'CA-AGC-ACA-GTG-NNN-TAC
TCG-AGT-TCG-TGT-CAC-NNN-ATG-GGC-C5'
Subtilisin
5'
Eco RI
3'
Bam HI
UB110 ori
pS4.5
322 ori
CAT
Sac I
Xma I
pΔ 166
M13 Mutagenesis
Ligate/Transform
Kpn I (152)
pV 152
166
Sac I
Xma I
Eco RI
Bam HI
p166
1. Kpn I / Klenow
2. Bam HI
Sac I / Bam HI
FRAGMENT 1
BluntI
Bam HI
FRAGMENT 2
Sac I
Bam H I
FRAGMENT 3
-156-
-gln-
5'
Sac I
GCA-GCC-GGT-AAC-CAA-GGT-ACC-TCC-GGG-AGC-3'
CGT-CGG-CCA-TTG-AGA-CCA-TGG-AGG-CCC 5'
-ser-
Ligate/Transform
156 166
p156/166

FIG.— 21

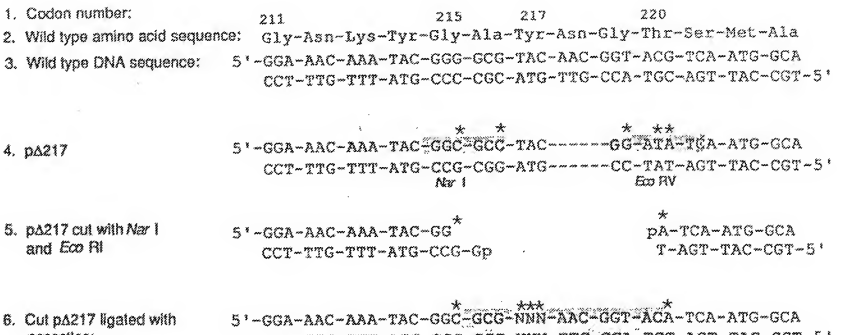


FIG.—22

0251446

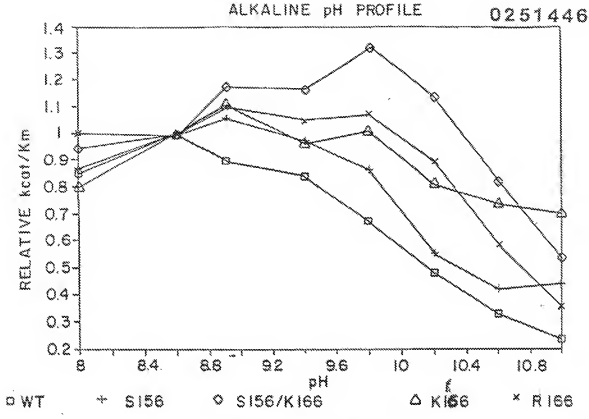


FIG. — 23A

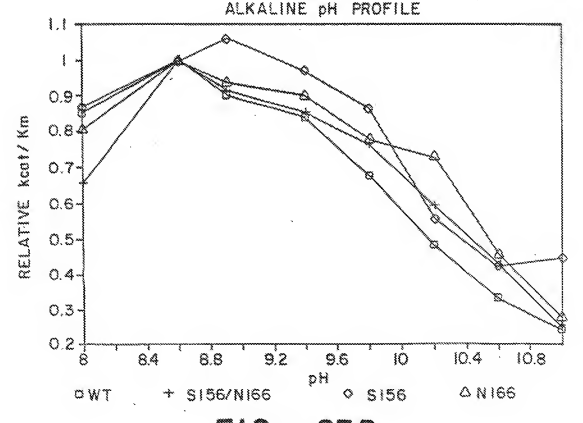


FIG. — 23B

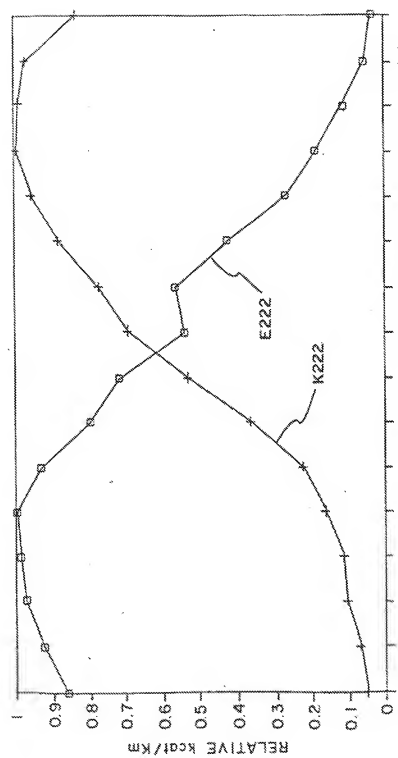

RELATIVE kcat/Km
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
7.4
7.8
8.2
8.6
9
9.4
9.8
10.2
pH
E222
K222

FIG. — 24

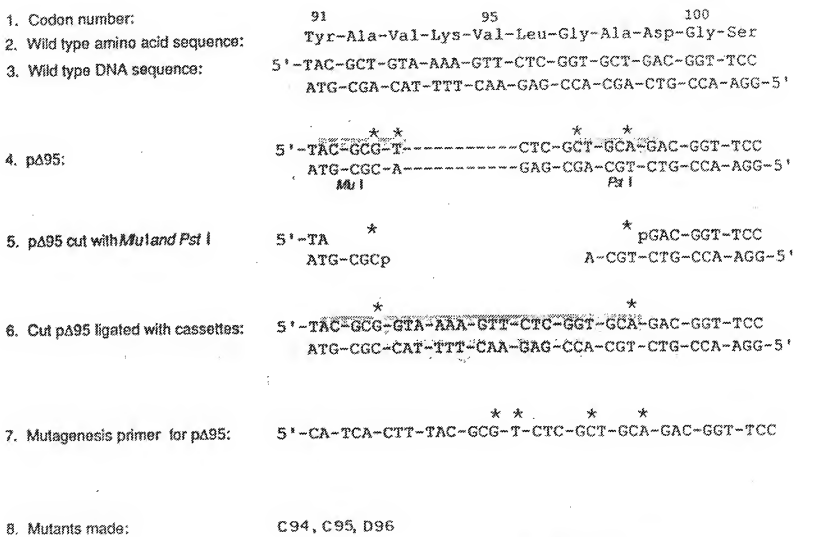


FIG.—25

0251446

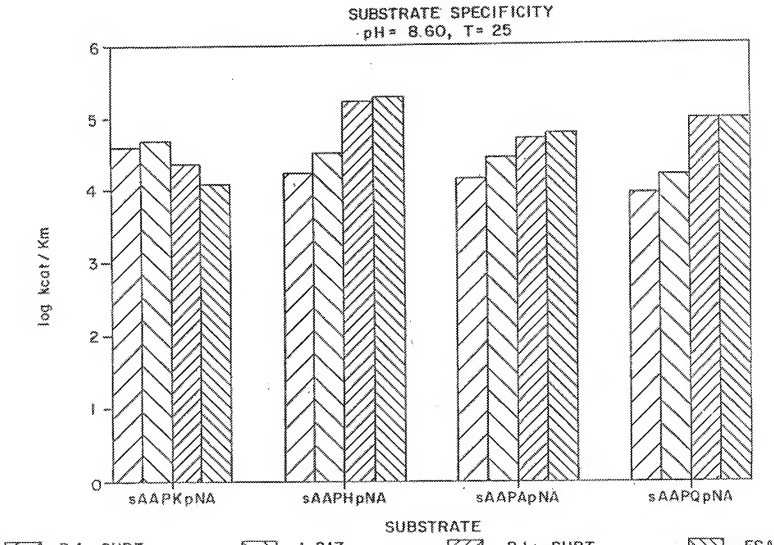

SUBSTRATE SPECIFICITY
pH= 8.60, T= 25
log kcat / Km
0
1
2
3
4
5
6
sAAPKpNA
sAAPHpNA
sAAPApNA
sAAPQpNA
SUBSTRATE
B.A. SUBT
L 217
B.L. SUBT.
FSAL

FIG.—26

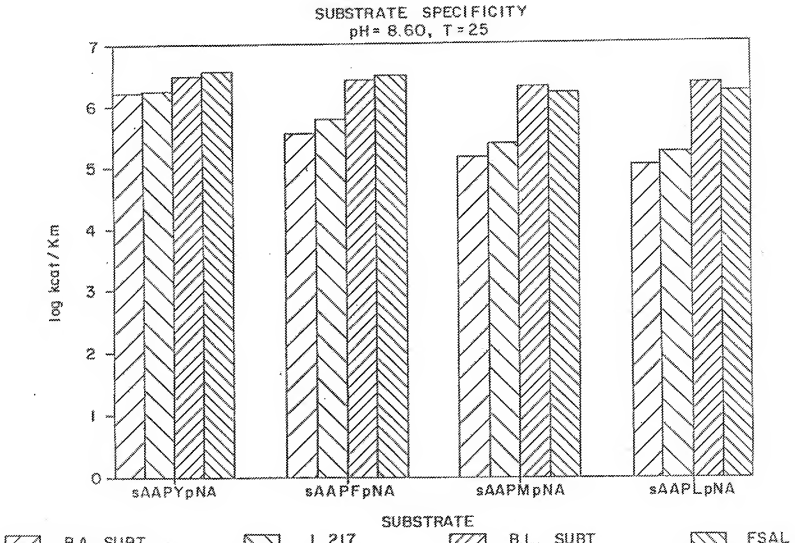


FIG.—27

0251446

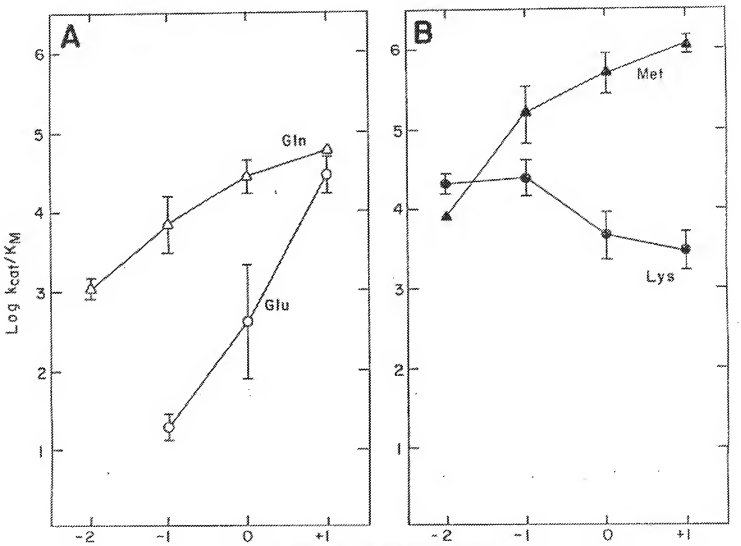


FIG.—28

0251446

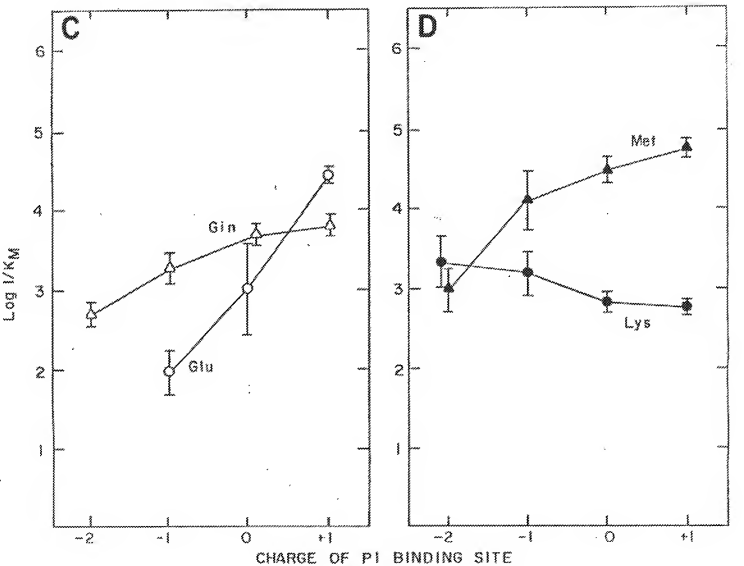


FIG.-28

0251446

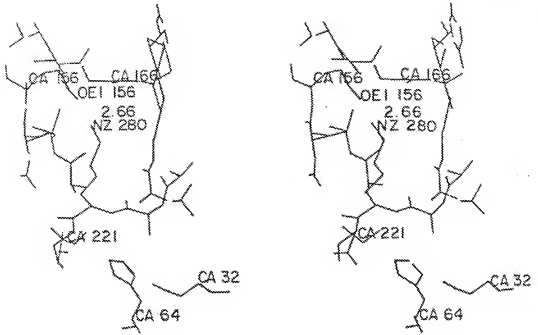


FIG.—29A

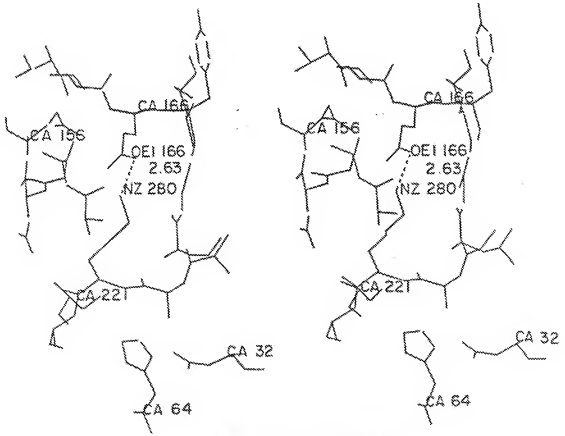


FIG.—29B

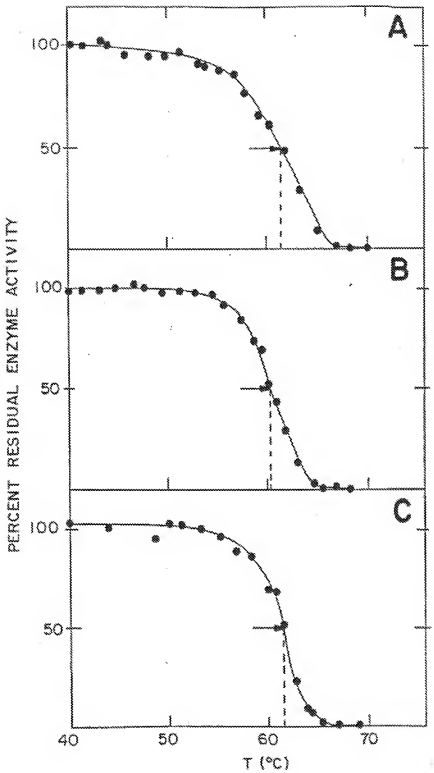

A
B
C
100
50
100
50
100
50
PERCENT RESIDUAL ENZYME ACTIVITY
40
50
60
70
T (°C)

FIG.—30

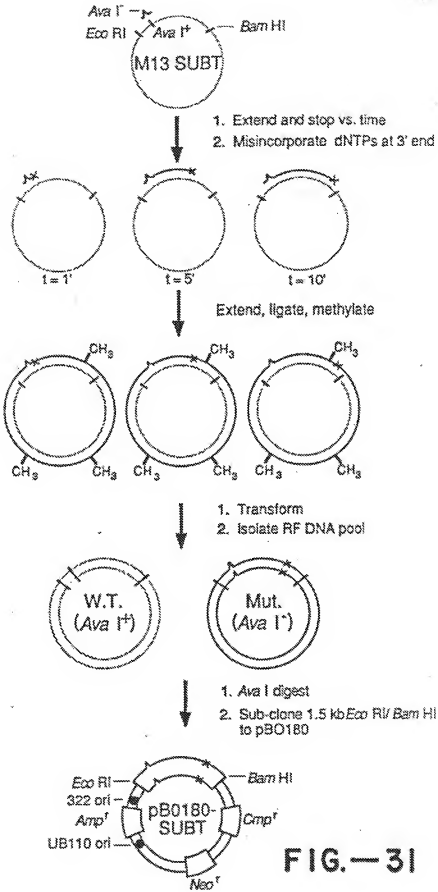

Ava I⁻
Eco RI
Ava I⁺
Bam HI
M13 SUBT
1. Extend and stop vs. time
2. Misincorporate dNTPs at 3' end
t = 1'
t = 5'
t = 10'
Extend, ligate, methylate
CH3
1. Transform
2. Isolate RF DNA pool
W.T.
(Ava I⁺)
Mut.
(Ava I⁻)
1. Ava I digest
2. Sub-clone 1.5 kb Eco RI/ Bam HI
to pBO180
Eco RI
322 ori
Amp^r
UB110 ori
pBO180-
SUBT
Bam HI
Cmp^r
Neo^r

FIG.—31

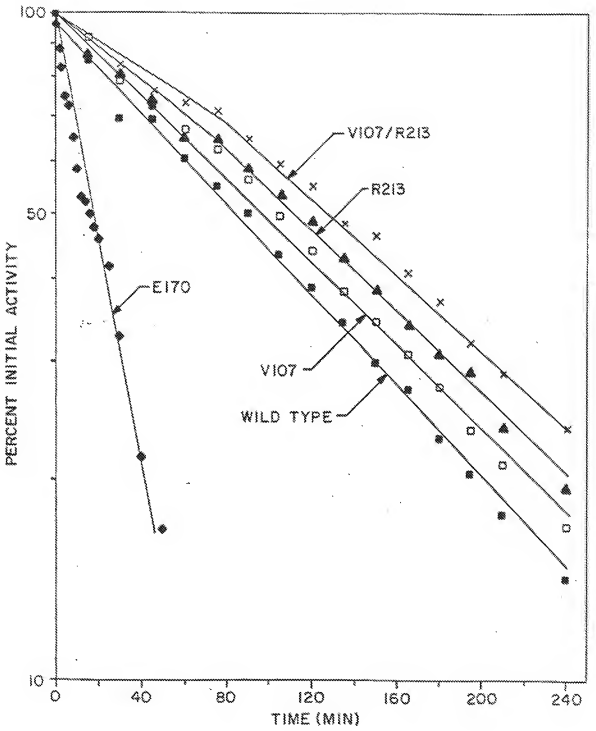

100
50
10
PERCENT INITIAL ACTIVITY
0
40
80
120
160
200
240
TIME (MIN)
V107/R213
R213
E170
V107
WILD TYPE

FIG. – 32

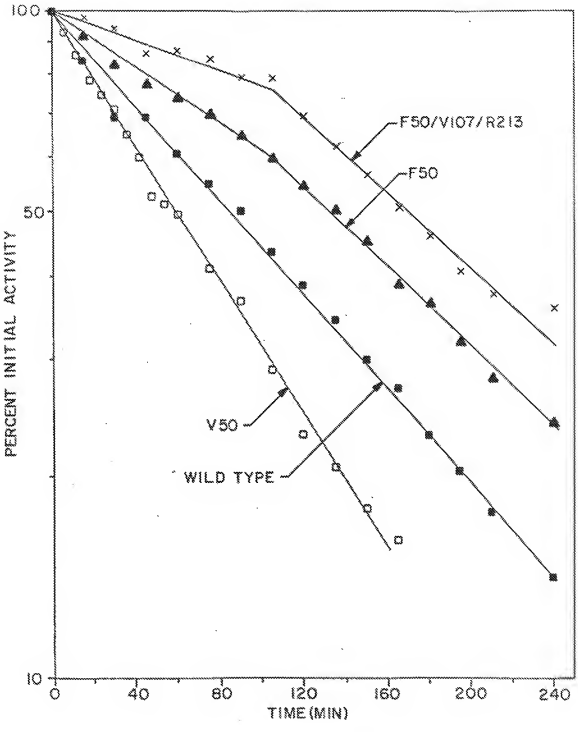

100
50
10
PERCENT INITIAL ACTIVITY
0
40
80
120
160
200
240
TIME (MIN)
F50/V107/R213
F50
V50
WILD TYPE

FIG.—33

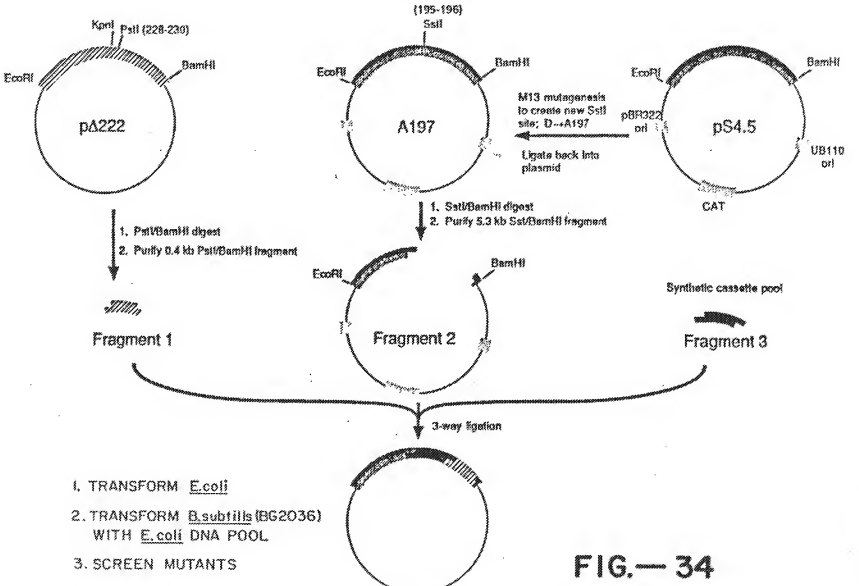
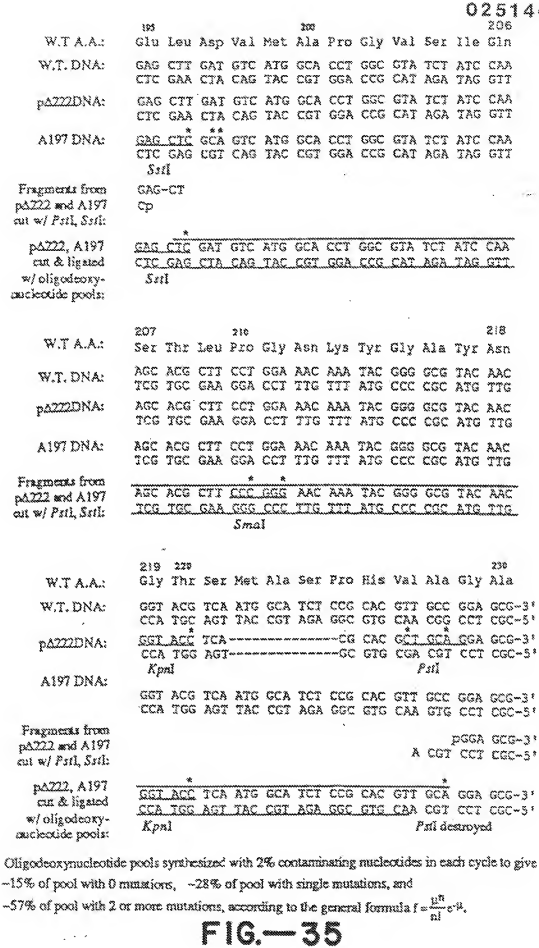


FIG.— 34

0251446



Oligodeoxynucleotide pools synthesized with 2% contaminating nucleotides in each cycle to give ~15% of pool with 0 mutations, ~28% of pool with single mutations, and ~57% of pool with 2 or more mutations, according to the general formula $f = \frac{u^n}{n!}e^{-u}$.

FIG.—35

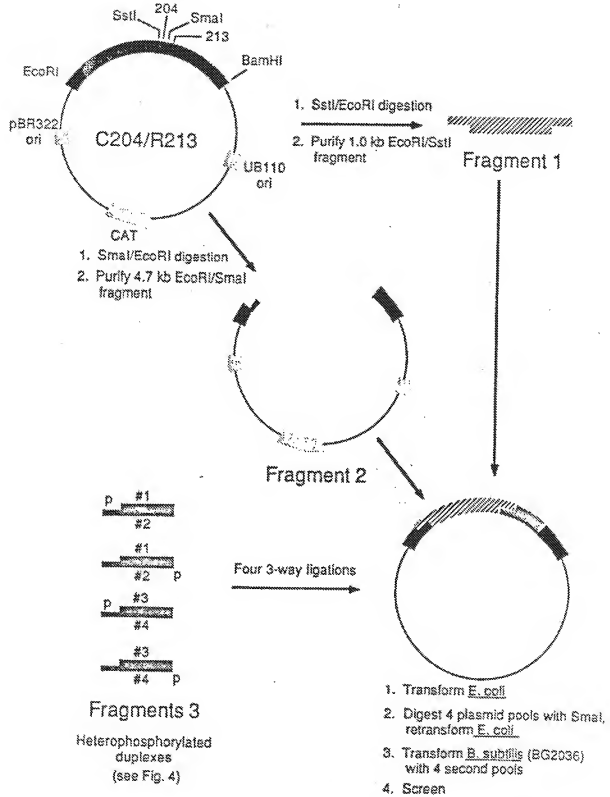

SstI
204
SmaI
213
EcoRI
BamHI
pBR322 ori
C204/R213
UB110 ori
CAT
1. SstI/EcoRI digestion
2. Purify 1.0 kb EcoRI/SstI fragment
Fragment 1
1. SmaI/EcoRI digestion
2. Purify 4.7 kb EcoRI/SmaI fragment
Fragment 2
p
#1
#2
#3
#4
Four 3-way ligations
Fragments 3
Heterophosphorylated duplexes
(see Fig. 4)
1. Transform E. coli
2. Digest 4 plasmid pools with SmaI, retransform E. coli
3. Transform B. subtilis (BG2036) with 4 second pools
4. Screen

FIG.—36

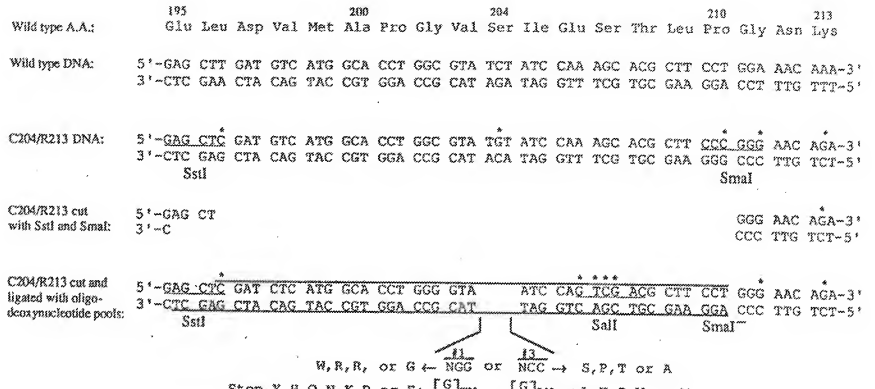


FIG.—37

0251446